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A test of *Eco*RI and *Hin*dIII restriction fragment length polymorphisms in assessing susceptibility for scrapie in US Suffolk sheep

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Abstract

We investigated the distribution of *Eco*RI restriction fragment length polymorphism (RFLP) and tested *Eco*RI and *Hin*dIII RFLPs to ascertain the occurrence of scrapie in Suffolk sheep. Genomic DNA samples were collected from 527 animals and typed for *Eco*RI RFLP by Southern blot analysis using the PrP gene clone probe (pNPU42). The average e1 frequency in Suffolk sheep was 64.4% and ranged from 51.5 to 82.2%, which was significantly higher than the average in Cheviot (32.3%) and Rambouillet sheep (17.7%). The association of histopathologic diagnosis with RFLPs was investigated using 82 animals exposed orally with scrapie inoculum in a double-blind, retrospective experimental design. There was a significant ($P < 0.01$) association between *Eco*RI RFLP and scrapie histopathologic diagnosis in Suffolk sheep with the e1e1 genotype. The use of the *Eco*RI RFLP phenotype for scrapie-diagnosis, however, was inconsistent for some individual cases. *Hin*dIII-produced RFLPs was not significantly related to scrapie diagnosis. © 1998 Elsevier Science B.V.

Keywords: Scrapie; Suffolk; RFLP

1. Introduction

Scrapie in sheep is the oldest known form of transmissible spongiform encephalopathy (TSE) and was described as early as 1732 (Schreuder, 1994). Similar-type diseases include Kuru, Creutzfeldt–Jakob, and Gerstmann–Straussler–Scheinker diseases in human, bovine spongiform encephalopathy (BSE) in cattle, feline spongiform encephalopathy (FSE) in cats and chronic wasting disease of mule deer and elk. TSE diseases are characterized by a long incubation period followed by progressive degeneration of the central nervous system. A unique characteristic of these disorders, whether sporadic, dominantly inherited, or acquired by infection, is the accumulation of a protease-resistant prion protein (PrP) in the brain of TSE-infected animals (Prusiner and Hsiao, 1994). PrP appears to be encoded by a single host gene.

Susceptibility of sheep to infection by various scrapie strains is influenced by genes identical to, or linked with, the PrP gene (Carlson et al., 1986;

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Hunter et al., 1989; Goldmann et al., 1990; Race et al., 1990). There are two types of PrP base sequence changes associated with scrapie susceptibility: Polymorphisms that lie within the coding region of the PrP protein gene [e.g., amino acid substitution polymorphisms resulting in changes at codons 112 (methionine or threonine), 136 (alanine or valine), 154 (arginine or histidine) and 171 (arginine, glutamine or histidine)] (Goldmann et al., 1990; Laplanche et al., 1993; Westaway et al., 1994) and polymorphisms outside the coding region (e.g., *Eco*RI- and *Hin*dIII-produced RFLPs) (Hunter et al., 1991).

The incidence of scrapie is found to be associated with amino acid polymorphisms at codons 136, 154 and 171 (Goldmann et al., 1991, 1994; Laplanche et al., 1993). In many breeds, including the Swaledale, Cheviot, Ile de France, Shetland, Scottish Halfbred and Bleu du Maine, most scrapie-positive sheep, either naturally infected or experimentally challenged, carry the Val-136 codon (Maciulis et al., 1992; Laplanche et al., 1993; Goldmann et al., 1994; Hunter et al., 1994). Westaway et al. (1994) found a strong linkage to scrapie predisposition with a PrP protein coding region polymorphism at codon 171 for glutamine homozygosity (Q/Q-171). Sheep that developed scrapie were homozygous for 171 (Q/Q-171). The non-scrapie sheep had genotypes either heterozygous for glutamine/arginine (Q/R-171) or homozygous for arginine (R/R-171). This association was also observed by O'Rourke et al. (1996) in natural occurring and in experimentally scrapie-infected (O'Rourke et al., 1997) flocks of US Suffolk sheep.

In British Cheviot sheep, Hunter et al. (1991) observed that *Eco*RI and *Hin*dIII-derived restriction fragment length polymorphisms were informative for predicting survival time of animals after exposure to the scrapie agent. Haplotype e1h2 was associated with an increased incidence of clinical scrapie, whereas e3h1 was associated with a decreased incidence. This same correlation was confirmed in US Cheviot sheep by Maciulis et al. (1992), and in Suffolk sheep in Japan (Ikeda et al., 1995).

Polymorphisms detected inside the PrP protein coding region, especially amino acid polymorphisms of PrP protein, appear to be informative predictors for scrapie susceptibility. Nevertheless, their use has limitations with respect to the incidence of scrapie in Suffolk sheep. The PrP variant of Val-136, which was strongly associated to the incidence of scrapie in many sheep breeds (Maciulis et al., 1992; Laplanche et al., 1993; Goldmann et al., 1994; Hunter et al., 1994), was rare in British and US Suffolk (Hunter et al., 1994; Goldmann et al., 1994; Westaway et al., 1994; O'Rourke et al., 1996) and low in Japanese Suffolk (Ikeda et al., 1995). The analysis of codon 171 of scrapie-affected Suffolk showed all to be Q/Q homozygous (Westaway et al., 1994; O'Rourke et al., 1996), whereas sheep homozygous for R/R-171 did not develop scrapie from either natural or experimental exposure to the agent (Laplanche et al., 1993; Goldmann et al., 1994). However, scrapie has been reported in Japanese Suffolk sheep homozygous for R/R-171 (Ikeda et al., 1995). Scrapie cases were also found in Japanese Suffolk heterozygous for Gln/Arg-171 (Ikeda et al., 1995) and in Scotland (Hunter et al., 1997). In a preliminary study conducted by our group, we observed that not all non-Q/Q-171 homozygous, experimentally infected sheep, were scrapie-negative (diagnosis based upon histopathology). Of the 5 Suffolk sheep in our study, one was R/Q-171 heterozygous and one R/R-homozygous; both were diagnosed as scrapie positive. O'Rourke et al. (1997) recently reported that 12 of the 75 Q/Q-171 experimentally infected Suffolk sheep remained scrapie free. These cases seem to indicate that the Gln-171 allele in the coding region may not be fully recessive for scrapie-resistance (Hunter et al., 1997); or alternatively, there may be additional polymorphisms within the PrP open reading frame or in the flanking regions (Ikeda et al., 1995; Hunter et al., 1996). Another possibly is that there is an unidentified gene that influences the control or modulates susceptibility to scrapie.

Hunter et al. (1997) reported that the anticipated occurrence of scrapie in a closed Suffolk flock in Scotland was not accompanied with a marked change in the frequency of the Q/Q-171 genotype. They observed an increase in the *Eco*RI haplotype with increased scrapie incidence. The *Eco*RI polymorphism lies on the side of the protein-coding exon (3′UTR) of the ovine PrP gene (Hunter et al., 1991; Westaway et al., 1994). This region transcribes RNA, but the message is not translated into PrP protein (Hunter et al., 1991; Westaway et al., 1994). Gold-

mann et al. (1991) found specific amino acid differences between RFLP genotypes. They reported that the genotype e1h2 has unique codons Val-136, Arg-154 and Gln-171, while e3h1 have Ala-136, Arg-154 and Arg-171 or Ala-136, His-154 and Gln-171. Ikeda et al. (1995) reported that there are links between the R-171 allele and particular patterns of *Eco*RI and *Hin*dIII restriction fragment length polymorphisms. These reports, as mentioned above, suggest that scrapie may not be a simple genetic disease as once thought, but one that is complicated by an interaction of genetic constitution and disease susceptibility. The contradictions currently being observed in different flocks of Suffolk sheep may be a clue that differences in gene expression may be a factor in scrapie susceptibility.

Suffolk sheep have the highest incidence of scrapie (Westaway et al., 1994). Based on the data reported by others concerning the properties of *Eco*RI RFLP in association with scrapie incidence and its relationship with amino acid polymorphisms in the coding region of the PrP gene (Hunter et al., 1991; Hunter et al., 1997; Westaway et al., 1994; Ikeda et al., 1995), and upon the results of our preliminary study, it appears that coding region polymorphisms may not unequivocally predict the incidence of scrapie in Suffolk sheep. This study investigates *Eco*RI RFLP polymorphic distributions of the PrP gene and evaluates the association of *Eco*RI and *Hin*dIII RFLP with scrapie susceptibility in Suffolk sheep.

2. Materials and methods

Genomic DNA was extracted by the phenol:chloroform procedure of Sambrook et al. (1989). Each genomic DNA sample was digested with the restriction enzymes, *Eco*RI or *Hin*dIII (Boehringer Mannheim, Indianapolis, IN), respectively, and then Southern blotted on a membrane (Zeta-Probe, Bio-Rad, Richmond, CA) following the procedure of Rigaud et al. (1987). After prehybridization for 12 h at 42°C in 6 × sodium chloride/sodium citrate solution (SSC), 10 × Denhart solution, the filter membranes were hybridized with a sheep DNA insert of a PrP coding-region marker probe (pNPU42, described by Hunter et al., 1991) obtained from AFRC & MRC Neuropathogenesis Unit, Edinburgh, Scotland in 50% formamide, 6 × SSC containing 100 μg of denatured salmon sperm DNA/ml and 10% dextran sulfate at 42°C for 24 h. The probe was labelled with [^{32}P]deoxycytidine triphosphate and a random-primed labelling kit (Boehringer Mannheim, Indianapolis, IN). Filters were washed twice for 15 to 30 min. in 2 × SSC containing 1% sodium dodecyl sulfate (SDS) at 55°C and then in a single 30 min wash in 0.1 × SSC and 1% SDS at 65°C.

2.1. RFLP distribution patterns

Blood samples or brain tissue samples were collected from 425 Suffolk sheep from 11 flocks (five flocks located in Texas, four flocks in Utah and two flocks in Iowa) and were used to investigate the distribution of *Eco*RI-produced RFLPs in US Suffolk sheep. Blood samples or brain tissue samples were also collected from 62 Cheviot sheep and 40 Rambouillet sheep. Genomic DNA was extracted form each sample and digested with *Eco*RI. The genomic digests were Southern-blotted to observe for fragment sizes 6.8 kb (e1) and 4.0 kb (e3) (Fig. 1). The *Eco*RI-produced polymorphism is located in the 3′ untranslated region (3′UTR) of the ovine PrP gene (Hunter et al., 1991). Chi-square was used to determine the statistical significance of differences of *Eco*RI polymorphic distribution between flocks and between breeds.

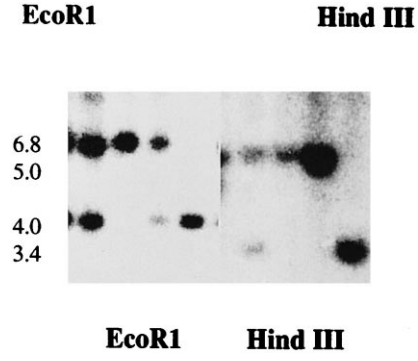

Fig. 1. Southern blot analysis of Suffolk sheep genomic DNA with a specific scrapie-associated fibrillar protein (PrP) DNA hybridization probe yielding *Eco*RI generated fragments of 6.8 (e1) and 4.0 (e3) kb, and *Hin*dIII-generated fragments of 5.0 (h1) and 3.4 (h2) kb.

2.2. Association of RFLP with scrapie diagnosis

A double-blind retrospective design was used to test the association of *Eco*RI and *Hin*dIII-produced RFLPs with scrapie diagnosis. DNA samples were collected from sheep ($n = 82$) that had been orally dosed with 30 ml of a 10% (w/v) pooled brain–spleen suspension that was third- or fourth-passage Suffolk scrapie agent prepared at the Scrapie Investigation Center in Mission, Texas (Foote et al., 1993). The inoculated sheep were observed until death or for at least 60 months after inoculation. Tissues were taken from all sheep at death. A section of brain tissue from each sheep that died during the project was sent to the USDA National Veterinary Services Laboratory, Ames, IA, for histopathological diagnosis. The remaining brain tissue was stored at -20°C for RFLP analysis. Genomic DNA was extracted. DNA sample was digested with *Eco*RI and Southern blotted as described above. Genomic DNA was also digested with *Hin*dIII, Southern blotted, and observed for fragment sizes 5.0 kb (h1) and 3.4 kb (h2) (Fig. 1). *Hin*dIII-produced polymorphisms lie on the side of the protein-coding exon of the ovine PrP gene (Hunter et al., 1991). The association of occurrence of *Eco*RI and *Hin*dIII haplotypes with histopathologic diagnosis was tested by Chi-square contingency analysis.

Eight Rambouillet sheep were also administered scrapie agent as described above. Genomic DNA samples were typed for RFLP polymorphism and brain sections were sent for histopathological diagnosis as described above.

The same double-blind retrospective design was used to examine the relevance of survival time of inoculated sheep with RFLP genotypes. The inoculated sheep were observed for clinical signs of scrapie until death or for at least 60 months after inoculation. Three sheep that survived past 60 months had to be culled at 2349, 3459 and 3453 days, respectively. Tissues were taken from all sheep at death for histopathological diagnosis. Histologic examination of sheep brains was conducted at the USDA National Veterinary Services Laboratory, Ames, IA to confirm scrapie. Calculations of survival time were based on scrapie death date, not onset of clinical signs, and all days to death were calculated from the time of inoculation (Foote et al., 1993). The difference in survival time between RFLP genotypes was measured by one-way analysis of variance (ANOVA).

3. Results and discussion

Restriction fragment length polymorphisms derived by *Eco*RI and *Hin*dIII digestions in US Suffolk sheep samples were similar to the reports of Hunter et al. (1989, 1991) and Maciulis et al. (1992) in the Cheviot sheep (Fig. 1). Listed in Table 1 is the distribution of *Eco*RI haplotypes. The e1 frequencies ranged from 51.1 to 82.5. between the Suffolk sheep flocks. The average occurrence (66.4%) of the e1 haplotype was significantly higher in Suffolk sheep than observed in US Cheviot and Rambouillet sheep. Based upon these data, one might expect an average of 45% (range from 26 to 68%) of Suffolk sheep in the US to be homozygous for the e1e1 genotype; however, there is considerable variation in the distribution of *Eco*RI RFLP between Suffolk flocks. These results are in agreement with the findings of Onodera et al. (1994) and Ikeda et al. (1995). They found that the distribution of molecular polymorphisms of PrP varied considerably between sheep breeds such as Suffolk and Corriedale and between flocks within the same breed.

The *Eco*RI-produced restriction fragment length polymorphisms were correlated with histopathological diagnosis of scrapie in inoculated animals (Table 2). The numbers of genotypes with e1e1, e1e3 and e3e3 RFLP genotypes were 38, 32 and 12, respectively. The number of positive diagnosis was 35, 15 and 3 for the respective phenotypes. Contingency table analysis indicated a significant ($P < 0.01$) association between *Eco*RI RFLP genotypes and histopathologic diagnosis. The e1 allele was associated with scrapie susceptibility and the e3 was associated with survival. Survival time for the e1e1 genotype sheep was significantly ($P < 0.01$) shorter than those of other genotypes. Ikeda et al. (1995) reported an association between the *Eco*RI RFLP and incidence of scrapie in Japanese Suffolk sheep. Hunter et al. (1997) also reported an increase in the disease-associated *Eco*RI haploid with the appearance of clinical scrapie in Scottish Suffolk. Chi-square analysis, on the other hand, indicated that there was no significant ($P > 0.05$) correlation with

Table 1
Distribution of *Eco*RI-produced restriction fragment length polymorphisms in some US Suffolk sheep flocks

Flock	Genotypes			Total	RFLP type frequency (%)	
	e1e1 6.8/6.8[d]	e1e3 6.8/4.0	e3e3 4.0/4.0		e1 6.8	e3 4.0
Suffolk						
Iowa-1	12	15	3	30	65.0	35.0
Iowa-2	16	20	3	39	66.7	33.3
Texas-1	15	14	1	30	73.3	26.7
Texas-2	18	10	5	33	69.7	30.3
Texas-3	10	23	5	38	56.6	43.4
Texas-4	17	23	5	45	63.3	36.7
Texas-5	13	19	12	44	51.1	48.9
Utah-1	3	13	0	16	59.4	40.6
Utah-2	23	36	8	67	61.2	38.8
Utah-3	28	14	1	43	81.4	18.6
Utah-4	26	14	0	40	82.5	17.5
Pooled				425	66.4[a] ± 3.0	
Cheviot	7	26	29	62	32.3[b]	67.7
Rambouillet	3	8	29	40	17.7[c]	82.5
Total				527		

[a,b,c] Means within column without common superscripts differ ($P < 0.05$).
[d] Denotes restriction fragment sizes (kb).

diagnosis of scrapie in *Hin*dIII-produced RFLP in our inoculated Suffolk sheep (Table 2). This observation is different from that reported for Cheviot sheep in Great Britain (Hunter et al., 1989, 1991) and in the USA (Maciulis et al., 1992). In all three studies, the h1h1 and h2h2 homozygous genotypes from *Hin*dIII digests were significantly associated with the incidence of scrapie.

The haplotypes for 32 of the 79 US Suffolk sheep typed with *Eco*RI-produced RFLPs (e1e1, e1e3 and e3e3) did not correspond with *Hin*dIII-produced RFLPs (h2h2, h1h2 and h1h1). In British Cheviot sheep, genotypes of the PrP gene typed with *Eco*RI-produced RFLP always correlated with *Hin*dIII-produced RFLP genotypes (e.g., *Eco*RI genotypes e1e1, e1e3 and e3e3 corresponds to *Hin*dIII genotypes h2h2, h1h2 and h1h1, respectively) (Hunter et al., 1989). The *Eco*RI RFLP homozygote was typed as either heterozygous or homozygous for the *Hin*dIII-produced RFLP. None of the Cheviot sheep investigated had the homozygous *Eco*RI genotype e1e1 or the homozygous *Hin*dIII h1h1 genotype (Maciulis et al., 1992). In this study, nine cases were observed where US Suffolk sheep were typed as e1e1 homozygote with *Eco*RI and h1h1 with *Hin*dIII and confirmed to be scrapie-positive by histopathological diagnosis.

In our study, the link between *Eco*RI RFLPs and the susceptibility to scrapie was not always consistent. As shown in Table 2, three of the 38 e1e1 genotype sheep were diagnosed as scrapie-negative, and 3 of the 12 e3e3 sheep were diagnosed as positive. In the heterozygous group, RFLP analysis was not predictive. One-half of the Suffolk sheep were diagnosed as positive and one-half as negative. This inconsistency was also observed in the Rambouillet sheep experimentally inoculated with scrapie. Seven of the eight sheep typed for *Eco*RI polymorphisms had e3e3 genotypes, and one was typed as an e1e3 heterozygote. Histopathology results indicated that three of the Rambouillet e3e3 homozygote were scrapie-positive. One e1e3 heterozygous and four e3e3 homozygous Rambouillet sheep were scrapie-negative. The inconsistencies between genotype and occurrence of scrapie may be the result of these RFLPs being situated outside the PrP gene coding region (Goldmann et al., 1990; Hunter et al., 1992).

RFLPs detected inside the PrP protein coding region, especially amino acid polymorphisms of PrP protein, appear to be very informative (Schreuder,

Table 2
The relationship of *Eco*RI and *Hin*dIII restriction fragment length polymorphisms (RFLPs) to scrapie incidence and survival time[b] in experimentally inoculated US Suffolk sheep

RFLP Genotype	*Eco*RI-produced RFLP			*Hin*dIII-produced RFLP		
	e1e1 6.8/6.8[a]	e1e3 6.8/4.0	e3e3 4.0/4.0	h2h2 3.4/3.4	h1h2 3.4/5.0	h1h1 5.0/5.0
Scrapie Positive	35	15	3	7	25	19
Scrapie Negative	3	17	9	10	14	28
Total	38	32	12	11	35	33
Chi-square ($df = 2$)		25.21		1.43		
P value		< 0.001		> 0.05		
Survival time (day)	895.8	1507.4[c]	1801.4[d]	1604.6	1209.6[c]	1258.6[d]
$\pm$SE	132.0	143.9	234.9	265.8	149.0	153.5
F-ratio		6.82			0.78	
P value		< 0.001			> 0.05	

[a] Denotes restriction fragment sizes (kb).
[b] Calculations of survival time were based on scrapie death date, not onset of clinical signs and all days to death were calculated from the time of inoculation.
[c] In this category, two sheep that were scrapie-negative were culled at 3459 and 3453 days, respectively, after inoculation.
[d] In this category, one sheep which was scrapie-negative was culled at 2349 days after inoculation.

1994). Amino acid polymorphisms of PrP protein are found to be associated with the susceptibility of scrapie in several sheep breeds (Goldmann et al., 1991, 1994; Laplanche et al., 1993). Goldmann et al. (1991) reported specific amino acid differences between RFLP genotypes. Genotype e1h2 has unique codons Val-136, Arg-154 and Gln-171, while e3h1 have Ala-136, Arg-154 and Arg-171 codons or codons Ala-136, His-154 and Gln-171. In Swaledale, Cheviot, Ile de France, Shetland, Scottish Halfbred and Bleu du Maine breeds, most scrapie-positive sheep, either naturally infected or experimentally challenged, carried the Val-136 codon (Maciulis et al., 1992; Laplanche et al., 1993; Goldmann et al., 1994; Hunter et al., 1994). However, the association between codon 136 and susceptibility to scrapie has not been substantiated in all scrapie-affected sheep breeds, including Suffolk sheep (Hunter et al., 1994; Westaway et al., 1994; Clouscard et al., 1995). The PrP variant of Val-136 was vary rare in British and US Suffolk (Hunter et al., 1994; Goldmann et al., 1994; Westaway et al., 1994; O'Rourke et al., 1996) and was quite low in Japanese Suffolk (Ikeda et al., 1995).

Polymorphism at codon 171 of the PrP gene appears to be a major genetic factor controlling the susceptibility to scrapie infection (Clouscard et al., 1995) and may be informative in predicating susceptibility to scrapie infection in Suffolk sheep. Westaway et al. (1994) found a strong linkage to scrapie predisposition with a PrP protein coding region polymorphism at codon 171 for glutamine homozygosity (Q/Q-171). The probability that Q/Q-171 is not related to scrapie susceptibility was extremely remote ($P = 0.000004$). The strong association of polymorphisms at codon 171 of the PrP gene with scrapie incidence was confirmed in studies on naturally infected Suffolk sheep in Japan (Ikeda et al., 1995) and in the United States (O'Rourke et al., 1996).

O'Rourke et al. (1997) recently demonstrated an association of susceptibility to scrapie disease with the Q/Q-171 genotype. Among the orally challenged US Suffolk sheep, those that developed scrapie were homozygous for glutamine at codon 171 (Q/Q-171). In our study, 18 of the 53 sheep that contracted scrapie were not e1e1 (Table 2). Part of the sheep included in the study of O'Rourke et al. (1997) were from Suffolk sheep reported in this study. Their results indicated that the coding region polymorphisms are more powerful predictors for scrapie susceptibility than outside the coding region. Twelve of the 75 Q/Q-171 homozygous, experimentally infected, Suffolk sheep were histopathologically negative (O'Rourke et al., 1997). Three of the 38 e1e1 homozygous, experimentally infected, Suffolk sheep were histopathologically negative in our study (Table 2). The *Eco*RI polymorphism lies on the side

of the protein-coding exon (3′UTR) of the ovine PrP gene (Hunter et al., 1991). This is the region transcribed into RNA but not translated into PrP protein (Hunter et al., 1991; Westaway et al., 1994). The disparity between our study and O'Rourke et al. (1997) seems to infer that differences in gene expression and scrapie susceptibility is not only due to amino acid polymorphisms in the PrP gene, but possible due to yet another unidentified gene or genes in the PrP gene family.

We conclude that the *Eco*RI RFLP is associated with scrapie susceptibility to oral infection in Suffolk sheep. The homozygous e1e1 is more related to the incidence of scrapie than heterozygous genotypes. The distribution of *Eco*RI RFLP indicates that many US Suffolk sheep appear to be at relatively high risk to scrapie infection. The use of *Eco*RI RFLP, however, to solely predict scrapie susceptibility in US Suffolk is currently limited by inconsistencies between individual RFLP haplotypes and scrapie-diagnosis in some individual cases.

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